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Generation of Transforming Growth Factor-α from the Cell Surface by an *O*-Glycosylation-independent Multistep Process*

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The precursor for transforming growth factor-α (TGF-α) is a membrane glycoprotein that can establish contact with epidermal growth factor/TGF-α receptors on adjacent cells or can be cleaved to release TGF-α that diffuses into the medium. Cleavage of pro-TGF-α occurs at Ala/Leu-Ala/Leu-Ala-Val-Val sites located at each end of the mature TGF-α sequence. To characterize the cleavage process of pro-TGF-α and the role of glycosylation in this process, we have introduced a pro-TGF-α expression vector in wild type Chinese hamster ovary (CHO) cells and in the mutant CHO cell clone *ldl*D that has a reversible defect in protein glycosylation. Analysis of metabolically labeled and cell surface-labeled products immunoprecipitated with antibodies directed against the extracellular TGF-α sequence and the cytoplasmic pro-TGF-α C-terminal domain shows that cleavage of pro-TGF-α in wild type CHO cells occurs in two steps. Both processing steps occur after pro-TGF-α reaches the cell surface. In the first step, pro-TGF-α rapidly ($t_{½}$ = 30 min) loses the amino-terminal segment that precedes the TGF-α sequence. In the second step, pro-TGF-α is cleaved at the carboxyl terminus of the TGF-α sequence releasing this factor into the medium. This second step is slow ($t_{½}$ = 2 h). The action of pancreatic elastase added to CHO-TGF-α cells mimics the first step but not the second one. Synthesis, cell surface exposure, rate of cleavage, and generation of bioactive TGF-α in *ldl*D-TGF-α cells are not markedly affected by the lack of *N*-acetylgalactosamine-dependent protein *O*-glycosylation or galactose-dependent glycan chain modification. The results indicate that, despite their similarity in amino acid sequence, the two cleavage sites that flank TGF-α may be processed with different kinetics which can lead to retention of pro-TGF-α on the cell surface.

Transforming growth factor-α (TGF-α)[1] is a mitogenic polypeptide first identified in the culture fluids of various tumor-derived, retrovirally transformed, and chemically transformed cells (1-3, for review, see Ref. 4) but expressed also in normal skin, pituitary, and decidua cells (5-7). TGF-α is structurally and functionally related to epidermal growth factor (EGF) (3, 7-10). These two factors are indistinguishable in their ability to bind to and activate the same receptor in mammalian cells (11, 12) but differ in their ability to interact with the avian receptor which has higher affinity for TGF-α than for EGF (13).

An important feature of TGF-α and related factors is that they are synthesized as part of transmembrane glycoprotein precursors. In addition to EGF (14, 15) and TGF-α (9, 10), other members of this family include vaccinia growth factor that is encoded by vaccinia virus (16-18), the products of two *Drosophila* neurogenic genes, *Notch* and *Delta* (19, 20), and the products of two genes, *lin-12* and *glp-1*, that determine cell fate in the nematode *Caenorhadditis elegans* (21-24). Colony-stimulating factor-1 and tumor necrosis factor-α are not members of the EGF family, but are also synthesized as part of membrane-bound precursors (25, 26).

The TGF-α precursor, pro-TGF-α, is a polypeptide of 159 (rat) or 160 (human) amino acids with a sequence of 23 hydrophobic amino acids that starts at residue 97 or 98 (9, 10) and acts as a membrane-spanning domain (27, 28). The 50-amino acid TGF-α sequence is located in the extracellular domain or pro-TGF-α, starting with residue 39 or 40. The proteolytic process that releases mature TGF-α from pro-TGF-α is inefficient under basal conditions in most cell types examined including tumor-derived cells, retrovirally transformed cells, and cells transfected with a TGF-α gene (29-34). As a result, cells release partially processed forms of TGF-α or retain pro-TGF-α on their surface.

Uncleaved pro-TGF-α expressed on the surface of one cell can establish contact with the EGF/TGF-α receptor on the surface of an adjacent cell and activate the receptor-associated tyrosine kinase activity (33, 34) and calcium uptake (33). Furthermore, cell-cell interactions mediated by the EGF receptor/pro-TGF-α pair can support cell adhesion associated with a mitogenic response.[2] This form of cell-cell stimulation might play an important role in developmental processes that depend on discreet cell-cell interactions, or targeting pro-TGF-α expressing cells to tissue sites rich in TGF-α receptor.

The events that convert pro-TGF-α from a membrane molecule to a diffusible autocrine/paracrine factor are poorly understood. These events could be the object of regulatory stimuli or the subject of defects that might lead to changes in the pattern of expression of the membrane-bound and secretory forms of TGF-α. Preliminary studies on the biosynthesis of pro-TGF-α have been reported (27, 29, 33, 34). However, the cellular location where pro-TGF-α is cleaved, the mechanism that allows pro-TGF-α to reach the cell surface un-

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[1] The abbreviations used are: TGF-α, transforming growth factor-α; EGF, epidermal growth factor; PBS, phosphate-buffered saline solution; CHO cells, Chinese hamster ovary cells; MEM, minimal essential medium.

[2] Anklesaria, P., Teixidó, J., Laiho, M., Pierce, J. H., Greenberger, J., and Massagué, J. (1990) *Proc. Natl. Acad. Sci. U. S. A.*, in press.

cleaved, and the role of post-translational modifications such as glycosylation in this process have been unknown. The present studies address some of these issues using as a model system CHO cells transfected with a pro-TGF-α gene. The results show that processing of pro-TGF-α occurs mainly after this molecule reaches the cell surface.The different kinetics of cleavage at the TGF-α flanking sites in the precursor provide an explanation for the accumulation of uncleaved pro-TGF-α on the surface of CHO cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Parental CHO (clone K1) (American Type Culture Collection) and *ldl*D cells (obtained from M. Krieger, MIT; Ref. 35) were grown in MEM-α medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) (complete medium). The pSV2-TGF-α vector was constructed by placing the *Sma*I fragment of plasmid 3B1b (10), which encodes the entire rat pro-TGF-α, under the control of a SV40 early promoter in an expression vector that also contains the mouse dihydrofolate reductase gene under the control of a separate SV40 promoter (36). Subconfluent cells in 100-mm dishes grown in complete medium were co-transfected with 5 μg of pSV2-TGF-α DNA and 0.5 μg of pZIP/neo DNA, which contains the bacterial transposon Tn5 neomycin resistance gene (37), as a calcium phosphate precipitate (38). Cells were incubated for 4 h at 37 °C with the plasmid DNA, and the medium was then removed. Cell cultures were incubated for 3 min with 2.5 ml of 15% (v/v) glycerol. Cells were left in regular medium for 24 h and then selected in the same medium in the presence of 1 mg/ml G418 (Geneticin; Gibco Laboratories) until antibiotic-resistant colonies appeared. Antibiotic-resistant colonies were ring-cloned and analyzed for TGF-α mRNA expression using a previously described RNase protection assay (39). Positive clones were cultured in the presence of stepwise increasing concentrations (up to 5 μM) of methotrexate to amplify TGF-α expression. The cell clones with the highest level of TGF-α expression as determined by biosynthetic labeling and immunoprecipitation (see below) were used in all the experiments described in this study. These clones were designated CHO-TGF-α (cl 10.7) (derived from parental CHO cells) and *ldl*D-TGF-α (cl α4) (derived from CHO *ldl*D cells), and were maintained in MEM-α medium lacking nucleotides and supplemented with 10% dialyzed fetal bovine serum.

Cell Labeling—For metabolic labeling of CHO-TGF-α cells, subconfluent cultures in 60-mm dishes were placed in serum-free and cysteine-free MEM containing 150 μCi per ml of [^{35}S]cysteine (Du Pont-New England Nuclear). After 15 min, the radioactive medium was substituted with complete MEM. For metabolic labeling of *ldl*D-TGF-α cells, sparse cultures grown in MEM-α supplemented with 10% dialyzed fetal calf serum were placed in MEM-α supplemented with 1% ITS$^+$ (a solution containing 0.63 mg/ml insulin, 0.63 mg/ml transferrin, 0.63 μg/ml selenous acid, 125 mg/ml bovine serum albumin, and 0.53 mg/ml linoleic acid; Collaborative Research) with or without 10 μM galactose and/or 100 μM *N*-acetylgalactosamine. *ldl*D-TGF-α cells were grown in this medium for 48 h and then metabolically labeled as described above. The indicated sugar supplements were present in the labeling medium and in the chase medium.

For incubations with elastase, metabolically labeled cell cultures were incubated for 15 min at 37 °C in MEM containing 300 μg/ml soybean trypsin inhibitor with or without 25 μg/ml porcine pancreatic elastase (Worthington).

Cell surface iodination was performed with cells in suspension. Cells were detached by incubation for 10 min at 37 °C in 20 mM sodium phosphate-buffered isotonic saline solution, pH 7.4 (PBS buffer), containing 1 mM EDTA, and recovered by centrifugation at 500 × *g* for 5 min. The samples were adjusted to contain equal numbers of cells in 1 ml of PBS buffer containing 0.2 mM phenylmethylsulfonyl fluoride. Iodination was carried out on ice by the addition of 0.5 mCi of $Na^{125}I$ (Amersham Corp.; 16 mCi/μg), 0.1 mg of lactoperoxidase, and 0.005% H_2O_2, with periodic mixing for 10 min. Cells were washed four times with PBS buffer containing 10 mM KI and 5 mM *N*-acetyltyrosine. These two components were also present in all buffers during immunoprecipitation of these samples.

Immunoprecipitations—At the indicated times after metabolic labeling or cell surface labeling, cells were rinsed with immunoprecipitation buffer (PBS buffer containing 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 200 kallikrein inactivating units/ml of aprotinin), scraped, and lysed in the same buffer containing 1% Nonidet P-40. Cell lysates were incubated with 5 mM dithiothreitol and 0.25% (w/v) sodium dodecyl sulfate for 20 min at 22 °C, followed by the addition of 10 mM *N*-ethylmaleimide prior to the addition of antibodies. This treatment increases the affinity of antigen for the anti-TGF-α antibodies. Anti-TGF-α antibodies (raised against a synthetic peptide corresponding to residues 71–88 of the rat pro-TGF-α sequence or residues 33–50 of rat mature TGF-α) (30) or anti-pro-TGF-α antibodies (raised against a mixture of two synthetic peptides corresponding to residues 138–151 and 145–159 of rat pro-TGF-α) (28) were used in the immunoprecipitations at a 1:30 dilution. Immunocomplexes were harvested with protein A-Sepharose and washed three times with PBS buffer containing 0.1% (v/v) Triton X-100, 0.02% sodium dodecyl sulfate, 0.5 mM phenylmethylsulfonyl fluoride, and 200 kallikrein inactivating units/ml of aprotinin, and once with PBS alone. The Sepharose beads were boiled in electrophoresis sample buffer and electrophoresed on 12 to 18% polyacrylamide gradient gels containing sodium dodecyl sulfate. Gels were then stained with Coomassie Blue and subjected to fluorography using Enlightning (Du Pont-New England Nuclear).

For immunoprecipitation of labeled secretory products, the culture medium collected at the end of the labeling period received 0.1 mM phenylmethylsulfonyl fluoride and was dialyzed against 0.1 M acetic acid, lyophilized to dryness, resuspended in immunoprecipitation buffer, and subjected to the immunoprecipitation protocol described above.

RESULTS

Generation of CHO Cell Lines That Express TGF-α—To generate cell lines for the study of TGF-α biosynthesis, we transfected the pSV2-TGF-α plasmid that contains the rat pro-TGF-α coding sequence transcribed under the control of an SV40 promoter (36). pSV2-TGF-α was transfected into wild type CHO cells and the mutant CHO cell clone, *ldl*D (35). Transfected cells were selected for resistance to the antibiotic G418 conferred by the pZIP/neo vector (37) that had been co-transfected with pSV2-TGF-α into the cells. To isolate cells with amplified pro-TGF-α expression, G418-resistant cell clones that had high levels of pro-TGF-α mRNA as determined by a RNase protection assay were selected for resistance to methotrexate conferred by overexpression of the dihydrofolate reductase gene present in the pSV2-TGF-α vector (36). Cell clones CHO-TGF-α (cl 10.7) and *ldl*D-TGF-α (cl α4) expressed the highest levels of pro-TGF-α and were used in this study.

pro-TGF-α Processing in Parental CHO Cells—Immunoprecipitation of lysates from CHO-TGF-α cells metabolically labeled for 15 min with [^{35}S]cysteine yielded an 18-kDa labeled product that was recognized by anti-pro-TGF-α antibodies directed against a cytoplasmic sequence of pro-TGF-α (28) as well as by anti-TGF-α antibodies (30) directed against an extracellular TGF-α sequence (Fig. 1, *A* and *B*). Antibody recognition of this 18-kDa product and all other labeled species described in this study was specific as determined by competition with an excess of the corresponding immunogenic synthetic peptides during immunoprecipitation (data not shown; Refs. 28, 30, and 33).

Within 15 min of metabolic chase with unlabeled medium, the 18-kDa pro-TGF-α form was almost completely converted into a 20–22-kDa product recognized by both antibodies (Fig. 1, *A* and *B*). Generation of the 20–22-kDa pro-TGF-α form was presumably due to the addition of carbohydrate during maturation of pro-TGF-α through the Golgi (Refs. 27, 29, and 33 and see below). *N*- and *O*-Glycosylation acceptor sites are located in the amino-terminal segment of pro-TGF-α that precedes the TGF-α sequence (27, 28, 31). The 20–22-kDa form was then chased into a 17-kDa form (Fig. 1, *A* and *B*). This form still retained the TGF-α epitopes suggesting that the loss of mass was due to the cleavage of the amino-terminal segment that precedes the TGF-α sequence. Conversion of the 20–22-kDa pro-TGF-α form to the 17-kDa pro-TGF-α

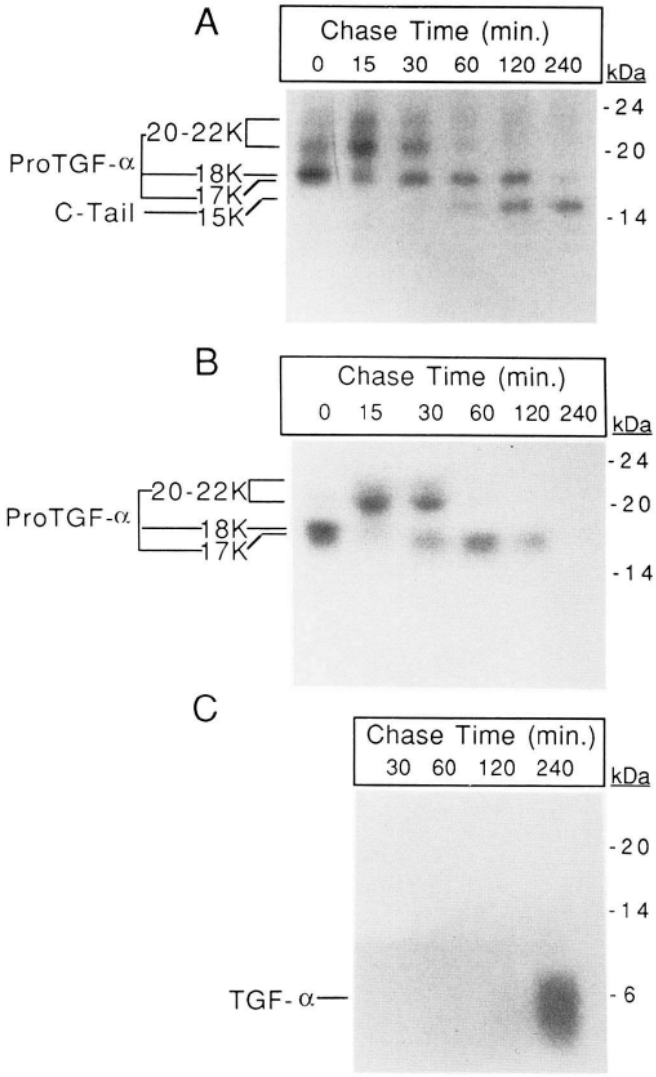

FIG. 1. **Biosynthesis of TGF-α in CHO-TGF-α cells.** CHO-TGF-α cells were metabolically labeled with [^{35}S]cysteine for 15 min. The label was then chased with regular unlabeled medium for the indicated time periods. Cell lysates (*A, B*) and media (*C*) were immunoprecipitated with antibodies against the carboxyl-terminal region of pro-TGF-α (*A*) or against a TGF-α sequence (*B, C*). Radiolabeled immunoprecipitated products were displayed by gel electrophoresis and fluorography of the resulting gels. The various products were identified as pro-TGF-α forms, mature TGF-α, or the transmembrane plus cytoplasmic domain (*C-Tail*) of pro-TGF-α, as described in the text. The position and molecular mass of protein standards are indicated. *K*, kilodalton.

form was relatively rapid, being half-maximal after 30 min of chase and essentially complete by 60 min.

Further processing of pro-TGF-α was slow and led to conversion of the 17-kDa form to a 15-kDa product. Conversion to this product was not complete until 4 h after synthesis (Fig. 1*A*). The 15-kDa product was not recognized by anti-TGF-α antibodies suggesting that it consisted of the transmembrane domain plus the cytoplasmic domain of pro-TGF-α left with the cell after release of mature TGF-α. Appearance of mature TGF-α in the medium was only detectable after pro-TGF-α was converted to the 15-kDa product (Fig. 1*C*).[3]

[3] The appearance of free mature TGF-α in the incubation medium reproducibly lagged behind the conversion of pro-TGF-α to the 15-kDa form. The reason for this lag is unknown but might be due to a slow diffusion of *de novo* cleaved TGF-α from the cell layer. Cell layer-associated TGF-α would be lost during the washes that preceded lysis and immunoprecipitation of cellular material.

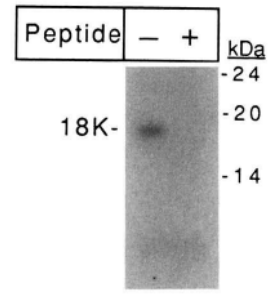

FIG. 2. **Pro-TGF-α exposed on the surface of CHO-TGF-α cells.** CHO-TGF-α cells were surface-labeled with ^{125}I. Cell lysates were immunoprecipitated with anti-pro-TGF-α antibodies in the absence (–) or presence (+) or excess (5 μM) synthetic peptide against which the antibodies had been raised. Immunoprecipitates were subjected to gel electrophoresis and autoradiography. *18K*, the specifically immunoprecipitated 18-kDa radiolabeled product.

The slow rate of cleavage of the 17-kDa pro-TGF-α form suggested that if pro-TGF-α were processed intracellularly it would have to be retained inside the cell for a relatively long time (up to 4 h). Alternatively, pro-TGF-α processing might take place after this molecule had reached the cell surface.

Two sets of experiments were performed to investigate these possibilities. First, CHO-TGF-α cells were surface-labeled with ^{125}I to determine if pro-TGF-α reached the cell surface before being cleaved. Immunoprecipitation of lysates from these cells yielded an 18-kDa labeled product specifically recognized by anti-pro-TGF-α antibodies (Fig. 2). This product is identified as the ^{125}I-labeled derivative of the 17-kDa pro-TGF-α form. Relative to the 17-kDa form, the 20–22-kDa pro-TGF-α form was not detectable, or was detectable only after prolonged autoradiographic exposure (see Fig. 5). We attribute this to the relatively short life of the 20–22-kDa product.

The second set of experiments was designed to probe the susceptibility of pro-TGF-α to cleavage by elastase. Elastase preferentially cleaves at alanine-, leucine-, and valine-rich sequences (40). Ala-Ala-Ala-/-Val-Val and Leu-Leu-Ala-/-Val-Val are the sequences and cleavage sites that flank the TGF-α sequence (9, 10). Elastase effectively cleaves the first one of these two sites in the pro-TGF-α extracellular portion that is released as a soluble product by certain transformed cells (30). To determine the sensitivity to elastase, CHO-TGF-α cell cultures that had been radioactively pulsed and then metabolically chased for various times were incubated with elastase for a short period. This treatment did not cleave the nascent 18-kDa pro-TGF-α form or the fully glycosylated 20–22-kDa pro-TGF-α in cells that had been chased for only 15 min (Fig. 3). This result was expected since pro-TGF-α labeled in this short pulse should still be mostly inside the cell and protected from exogenous elastase action. Thus, this experimental condition served as a negative control to indicate that elastase action was effectively stopped before preparation of cell lysates. However, the metabolically labeled 20–22-kDa pro-TGF-α form became accessible to cleavage by elastase 30 min after synthesis (Fig. 3). Elastase converted most of this form to a 17-kDa product. Interestingly, elastase action led to accumulation of this product with no further cleavage. The metabolically generated 17-kDa product was also resistant to exogenous elastase throughout the course of the chase (Fig. 3) suggesting that the action of exogenous elastase mimicked the first pro-TGF-α cleavage event but not the second one.

pro-TGF-α Processing in Glysosylation-defective Cell Mutants—Next, we examined the role of glycolysation on the biosynthesis and processing of pro-TGF-α in *ldl*D-TGF-α

cells. The phenotype of *ldl*D cells has been described in detail (35). Briefly, *ldl*D cells are deficient in UDP-galactose/UDP-*N*-acetylgalactosamine-4-epimerase and, therefore, cannot synthesize galactose or *N*-acetylgalactosamine from glucose. Under normal culture conditions with glucose as the only sugar substrate, the defect in *ldl*D cells leads to formation of glycoprotein cores lacking mucin type *O*-linked glycan chains whose synthesis is initiated by linkage of GalNAc to a serine or threonine residue. GalNAc added to the medium of *ldl*D cells is converted to nucleotide sugar by a salvage pathway that allows initiation of *O*-linked glycan chain synthesis to occur. However, in the absence of added galactose, *ldl*D cells cannot complete the synthesis of *O*-linked and certain *N*-linked glycan chains. Addition of both GalNAc and galactose is necessary to obtain the normal glycosylation phenotype in *ldl*D cells (35).

Fig. 4*A* shows that, in the presence of added GalNAc and galactose, *ldl*D-TGF-α cells exhibited a pro-TGF-α biosynthetic pattern similar to that of CHO-TGF-α cells. Thus, galactose- and GalNAc-supplemented *ldl*D-TGF-α cells synthesized pro-TGF-α as an 18-kDa product that was rapidly converted to a 20–22-kDa form followed by conversion to a 17-kDa form and then a 15-kDa product. The kinetics of these last two steps were similar ($t_{½}$ = 60 min), which contrasts with the different kinetics of these two steps seen in CHO-TGF-α cells. A 22–24-kDa labeled product immunoprecipitated from *ldl*D-TGF-α cells in these experiments (Fig. 4) was nonspecific as determined by its presence in immunoprecipitates obtained with excess immunogenic synthetic peptide (data not shown).

In the absence of added galactose and GalNAc, *ldl*D-TGF-α cells synthesized pro-TGF-α as a 17-kDa product that was converted to a 19-kDa form rather than the 20–22-kDa form seen in the wild type CHO cells (Fig. 4*C*). A 19-kDa form was also produced in *ldl*D-TGF-α cells supplemented with galactose but not GalNAc. These results suggested that both *O*-linked glycan chains and galactose-independent *N*-linked glycan chains were added to newly synthesized pro-TGF-α in wild type CHO cells, with the *O*-linked chains being responsible for most of the electrophoretic shift from 19-kDa to 20–22-kDa forms during pro-TGF-α synthesis.

The *O*-glycosylation defect expressed in *ldl*D-TGF-α cells in the absence of GalNAc did not have a major effect on the rate of formation of the 17-kDa pro-TGF-α form (Fig. 4), its accumulation on the cell surface as detected by surface labeling with ^{125}I (Fig. 5), or the rate of pro-TGF-α cleavage and appearance of TGF-α in the medium (Fig. 4 and Fig. 6, *A–D, lanes 1*) as compared to *ldl*D-TGF-α cells cultured with sugar supplements. To determine whether normal *O*-linked or *N*-linked carbohydrates were required for generation of a bioactive TGF-α conformation, we tested the ability of TGF-α to bind to EGF receptors. TGF-α produced by *ldl*D-TGF-α cells cultured with or without sugar supplements was incubated with EGF receptor-rich A431 cells. TGF-α produced under any of these culture conditions was able to bind to EGF receptors, and binding was specifically inhibited by the presence of an excess EGF (Fig. 6, *A–D, lanes 2–5*).

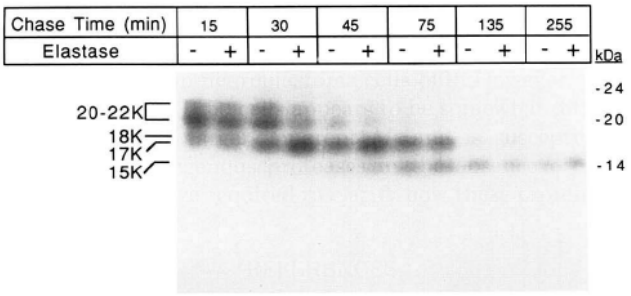

FIG. 3. **Susceptibility of pro-TGF-α to exogenous elastase.** CHO-TGF-α cells metabolically labeled with [^{35}S]cysteine for 15 min were chased with regular unlabeled medium for the indicated time periods. Porcine pancreatic elastase (25 μg/ml) was present during the last 15 min of the chase in the indicated (+) samples. Cell lysates were immunoprecipitated with anti-pro-TGF-α antibodies and displayed by electrophoresis and fluorography.

DISCUSSION

The present studies address several aspects of the processing of pro-TGF-α in mammalian cells. Our results indicate that generation of soluble TGF-α occurs after pro-TGF-α reaches the cell surface in a process that involves two cleavage events that can have markedly different kinetics.

Evidence to support these conclusions is provided by the combined results of metabolic labeling, surface labeling, and elastase treatment experiments. The results of metabolic labeling experiments in CHO-TGF-α cells show that newly synthesized pro-TGF-α becomes susceptible to cleavage by added elastase with kinetics characteristic of the movement of a membrane glycoprotein to the cell surface. The results also show that the first step of pro-TGF-α cleavage occurs as this molecule becomes exposed on the cell surface or soon thereafter. This first step results in trimming of the pro-TGF-α amino-terminal region. We infer that the initial loss of mass is due to trimming of the amino-terminal region because it occurs without loss of TGF-α epitopes and is mimicked by elastase. Accumulation of pro-TGF-α on the cell surface is clearly indicated by the detection of abundant 17-kDa form by extracellular radiolabeling. The second step of pro-TGF-α cleavage results in the loss of membrane-bound TGF-α epitopes and the appearance of mature TGF-α in the medium. This step occurs with very slow kinetics after pro-TGF-α reaches the surface of CHO-TGF-α cells. The results do not

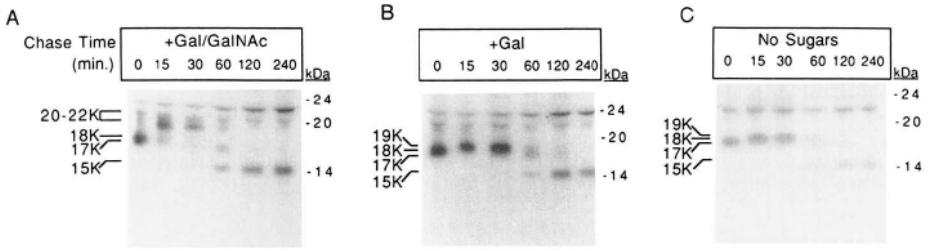

FIG. 4. **Biosynthesis of TGF-α in *ldl*D-TGF-α cells.** *ldl*D-TGF-α cells were metabolically labeled with [^{35}S] cysteine for 15 min. The label was then chased with regular unlabeled medium for the indicated time periods. Galactose and GalNAc were present in the media throughout the experiments as indicated. Cell lysates were immunoprecipitated with antibodies against the carboxyl-terminal region of pro-TGF-α. The resulting immunoprecipitates were subjected to gel electrophoresis and fluorography to display the labeled pro-TGF-α species.

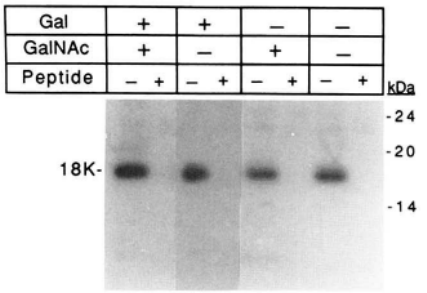

FIG. 5. **Cell surface pro-TGF-α species on the surface of *ldl*D-TGF-α cells.** *ldl*D-TGF-α cells grown in the presence of the indicated sugar supplements were surface-labeled with ^{125}I. Cell lysates were immunoprecipitated with antibodies against the carboxyl-terminal region of pro-TGF-α in the absence (−) or presence (+) of competing synthetic peptide immunogen. Samples were then subjected to electrophoresis and autoradiography.

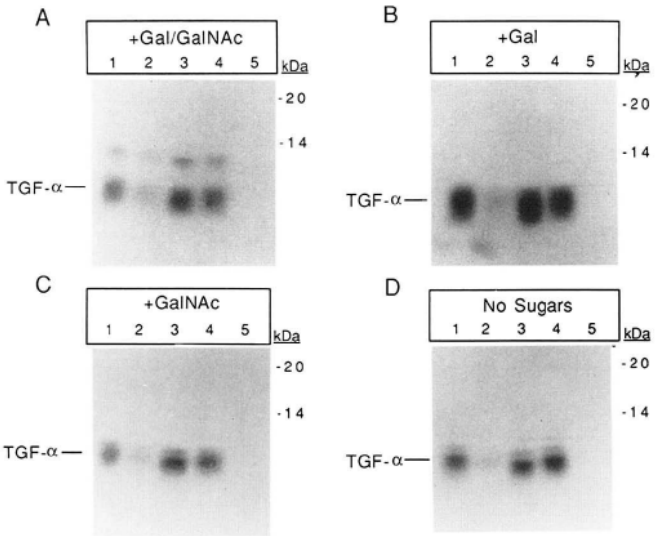

FIG. 6. **Production of bioactive TGF-α by *ldl*D-TGF-α cells expressing a protein glycosylation defect.** *ldl*D-TGF-α cells grown in the presence of the indicated sugar supplements were metabolically labeled with [^{35}S]cysteine for 20 min and chased with unlabeled medium for 5 h. The chase medium was collected and divided in three aliquots. One aliquot was prepared for immunoprecipitation without any additional treatment (*lanes 1*). The other two aliquots were incubated for 3 h at 4 °C with a sparse layer of A431 cells alone (*lanes 2* and *3*) or in the presence of 0.75 μM EGF (*lanes 4* and *5*). At the end of this incubation, the medium (*lanes 2* and *4*) and the A431 cells (*lanes 3* and *5*) were prepared for immunoprecipitation. All samples were immunoprecipitated with anti-TGF-α antibodies, electrophoresed, and subjected to fluorography. Note that regardless of the culture conditions, binding of *ldl*D-TGF-α cell-derived TGF-α to A431 cells was quantitative and was completely inhibited by the presence of excess EGF.

exclude the possibility that final cleavage of pro-TGF-α occurs intracellularly by internalization of this molecule from the cell surface.

Soluble TGF-α is generated by cleavage of pro-TGF-α at the carboxyl side of residues Ala^{38} and Ala^{88} (3, 9, 10). The amino acid sequences around the cleavage sites in rat pro-TGF-α are Ala-Ala-Ala^{38}-Val-Val and Leu-Leu-Ala^{88}-Val-Val, respectively (9, 10). The leucine-, alanine-, and valine-rich nature of both sequences predicts that they should be good substrates for elastase-like enzymes (40). Indeed, elastase has been previously shown to effectively cleave the TGF-α amino-terminal site in the pro-TGF-α extracellular portion released as a soluble product by transformed cells (30). However, the present studies suggest that the Ala^{88} site is resistant to cleavage by exogenous pancreatic elastase as well as by the cellular pro-TGF-α processing enzyme(s) in CHO-TGF-α cells. Resistance to cleavage at this site leads to accumulation of pro-TGF-α as a ccll surface protein. Resistance to cleavage might be due to the proximity of this site to the transmembrane sequence that starts at residue 97 following a Lys-Lys sequence (9, 10). The possibility that cleavage at Lys-Lys^{96} must occur first to render the Ala^{88} site accessible to cleaving enzymes is unlikely because pro-TGF-α forms mutated at and around residue 88 are not cleaved when they are expressed in various cell types including the parental CHO cells used here (33, 34).[4]

Certain cell lines release into the medium a partially processed pro-TGF-α form that consists of the TGF-α sequence extended with the heterogeneously glycosylated amino-terminal segment of pro-TGF-α (30–32). This segment is 16 or 38 amino acid residues long depending on whether the signal sequence is cleaved or not (9, 10).[5] The existence of this soluble form indicates that in some cell types cleavage at Ala^{88} can occur before cleavage at Ala^{38}. Production of a soluble form larger than TGF-α was observed in *ldl*D-TGF-α cells (Fig. 6) but not in CHO-TGF-α cells (Fig. 1*A*). In galactose plus GalNAc-supplemented *ldl*D-TGF-α cells, this form migrates on electrophoresis gels as a 10-kDa product. Production of this form by *ldl*D-TGF-α cells but not CHO-TGF-α cells could be explained by the observation that in the former cell line the kinetics of cleavage at both TGF-α-flanking sites are very similar, allowing TGF-α to abandon the cell surface before their N-terminal extension has been trimmed. These observations indicate cell-specific differences in the relative ability to cleave pro-TGF-α at the two TGF-α flanking sites and leave open the possibility that cleavage at each of these two sites is mediated by separate enzymes.

The role of *N*-glycosylation in the biosynthesis of pro-TGF-α has been determined using the inhibitor of co-translational *N*-linked glycan chain addition, tunicamycin. Tunicamycin interferes with the processing of pro-TGF-α in retrovirally transformed rat embryo fibroblasts (31) but not in transfected CHO cells (27), suggesting that tunicamycin action interferes with certain cell-specific pro-TGF-α processing activities. No specific inhibitors of protein *O*-glycosylation have been available to date to probe the role of this modification in the biosynthesis and activity of glycoproteins. However, the mutant CHO *ldl*D cell line provides an ideal system to determine the role of *O*-glycosylation because depending on the culture conditions these cells either display their inability to initiate biosynthesis of *O*-linked mucin-type glycan chains or they bypass this defect (35). Our results show that the rate of biosynthesis and cleavage of pro-TGF-α, and the production of a bioactive TGF-α conformation are not markedly altered in *ldl*D cells expressing the defect in *O*-glycosylation. Two other glycoproteins, human chorionic gonadotropin and apolipoprotein E, have been described whose rate of synthesis in *ldl*D cells is not affected by lack of *O*-glycosylation (41, 42). However, both these glycoproteins are soluble secretory products, not membrane components. The present results contrast with the requirement of *O*-glycosylation for normal biosynthesis of the other four different membrane glycoproteins examined to date. Krieger and co-workers (35, 42) have found that expression of low density lipoprotein receptor, decay

[4] J. Teixidó, D. C. Lee, and J. Massagué, unpublished work.

[5] Evidence suggests that the pro-TGF-α signal sequence is cleaved in CHO cells (34) but not in other cell lines (31) or in pro-TGF-α translated *in vitro* in the presence of dog pancreas microsomes (28). These apparent differences in processing may result from cell-specific differences in glycosylation of sites found near the signal peptide cleavage site (9, 10).

accelerating factor, and Epstein-Barr virus major antigen glycoprotein in *ldl*D cells leads to rapid degradation of these membrane glycoproteins when they reach the cell surface after synthesis, whereas the non-*O*-glycosylated interleukin-2 receptor is missorted or retained intracellularly never reaching the surface of *ldl*D cells (43). Thus, pro-TGF-α represents an exception among cell surface glycoproteins examined to date in that its sorting, expression, and processing do not seem to be affected by *O*-glycosylation.

We speculate that the stepwise nature of pro-TGF-α processing might be the object of regulatory mechanisms that would control the rate of conversion of this molecule from a membrane component to a diffusible autocrine/paracrine factor. It will be of interest to identify the enzymes involved in this conversion and the factors that may regulate their activity.

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